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Mechanism of Lysine Overproduction by a Yeast Mutant Resistant to a Lysine Sulfur Analog

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S-(β-Aminoethyl)-L-cysteine(SAEC) resistant mutants were induced from the wild-type strain of *Candida pelliculosa* by mutagenesis. Almost all resistant mutants excreted lysine into medium, and the lysine productivity increased by repeated mutation. Mechanism of lysine overproduction was investigated with the most potent producer, strain SR-V-1263. DL-α-Aminoadipate, a precursor of of lysine, stimulated extracellular accumulation of lysine by the mutant strain, but did not affect intracellular lysine level. The wild-type strain remarkably accumulated lysine extracellularly and intracellularly, when grown in the α-aminoadipate-medium, suggesting that both strains are not different in the permeability of lysine.

Homocitrate synthase activity of the cell-free extract of SR-V-1263 mutant was less sensitive than the wild-type enzyme to feedback inhibition by L-lysine and L-SAEC. The mutant strain could dissimilate lysine well as a sole nitrogen source as well as the wild-type strain.

Thus, it is concluded that the overproduction of lysine by SR-V-1263 mutant is attributed to of a feedback inhibition of homocitrate synthase by L-lysine, but not to a decrease in its abillty release of degrading intracellular lysine.

KEY WORDS: *Candida pelliculosa*/ *S*-(β-Aminoethyl)-L-cysteine(SAEC)/ Mutagenesis/ Extracellular and intracellular/ Homocitrate synthase/ Feedback inhibition/

INTRODUCTION

L-Lysine is one of the most important amino acids as a mammal nutrient. Various attempts have been made to produce microbial L-lysine with microorganisms because of its nutritional importance[1,2] and deficiency in cereal foods.[3-5] L-Lysine is industrially produced by several bacterial auxotrophs of glutamic acid-producing bacteria, *e.g.*, homoserine or threonine auxotrophs of *Corynebacterium glutamicum*[6] and *Brevibacterium flavum*.[6,7]

Since the studies by Adelberg and Cohen,[8,9] various amino acids analog-resistant mutants of bacteria have been induced to investigate the regulatory process in the bacterial cells,[10] and some of them are used to produce amino acids in practice.[11-13] Sano and Shiio[14] reported that *S*-(β-aminoethyl)-L-cysteine (SAEC) resistant mutants of *B. flavum* produce lysine effectively. Little information, however, is available about the lysine production by yeast resistant mutants with a few exceptions.

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In previous papers[15,16] we reported the isolation of SAEC-resistant mutants of *Candida pelliculosa* excreting lysine effectively, and the effect of cultural conditions on lysine production by the mutants. We have found that the most potent producer, SR-V-1263, accumulated more than 3 mg/ml of lysine under the best conditions. The present paper describes the mechanism of lysine overproduction by strain SR-V-1263.

EXPERIMENTAL PROCEDURES

Materials. *S*-(β-Aminoethyl)-L-cysteine·HCl was synthesized from L-cysteine and ethyleneimine by a modification[17] of the method of Cavallini *et al.*[18] Amino acids were products of Ajinomoto Co., Tokyo. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was purchased from Wako Chemicals, Osaka, and vitamins from Iwaki Seiyaku Co., Tokyo and L-lysine decarboxylase of *Bacterium cadaveris* from Kyowa Hakko Kogyo Co., Tokyo. Acetyl-CoA was a product of Sigma Chemical, St. Lowis, Mo., U.S.A. DL-α-Aminoadipic acid and other chemicals were analytical grade reagents and obtained from Nakarai Chemicals, Kyoto.

Microorganisms. *C. pelliculosa* (IFO 0707) and strain SR-V-1263 were used throughout the experiments.

Induction of SAEC-resistant mutants. Mutation experiments were carried out as described previously.[15]

Lysine production. Screening of lysine-excreting mutants and lysine accumulation by strain SR-V-1263 were carried out as described previously.[16] Intracellular and extracellular lysine were determined manometrically with L-lysine decarboxylase of *B. cadaveris* or by the acid ninhydrin method.[19]

Preparation of cell-free extracts. The wild-type and SR-V-1263 mutant were cultured in 1 liter of Lingens-Oltmanns' complete medium[20] placed in a 2-liter flask with reciprocal shaking at 28°C for 24 hr. The cells harvested by centrifugation were washed twice with 0.85 % NaCl solution at 4°C. The washed cells were suspended in 0.1 M potassium phosphate buffer (pH 6.2) containing 1 mM dithiothreitol and 20 % glycerol and disrupted completely in a Vibrogen cell mill with glass beads. The paste obtained was mixed with the same buffer followed by centrifugation. The supernatant solution was dialyzed overnight against 0.05M of the same buffer, and used as a crude enzyme.

Homocitrate synthase assay. The enzyme was assayed according to the method of Srere *et al.*[21] for the measurement of citrate synthase activity, based on the determination of CoA formed. The assay mixture contained 100 μmol of potassium phosphate buffer (pH 7.8), 50 μmol of α-ketoglutarate, 0.17 μmol of acetyl-CoA and enzyme in a final volume of 1.0 ml. The mixture was incubated at 30°C for 10 min the reaction was terminated by the addition of 3 ml of ethanol. After centrifugation to 0.5 ml of the supernatant solution was added 1.0 ml of 0.25 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) solution, and the absorbance at 412 nm was measured with a Beckman Specta-20 spectrophotometer (a light path, 1.0 cm). In a blank, α-ketoglutarate was replaced by water. No increase in absorbance was observed in a blank, indicating that acetyl-CoA deacylase is not present in *C. pelli-*

culosa. Under the conditions used, the reaction proceeded linearly with time, provided that ΔA_{412} did not exceed 0.5. The initial velocity was also proportional to the enzyme concentrations.

RESULTS

Induction and Screening of Lysine-Excreting Mutants

Attempts were made to induce SAEC-resistant mutants from the wild-type strain by ultraviolet irradiation and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) treatment. Among the resistant mutants induced by the first mutation (NTG treatment), strain SR-I-69 excreting about 0.8 mg/ml of lysine in screening medium B[16] was chosen. The lysine productivity of this strain was increased by further mutations. Fig. 1 shows distribution patterns of lysine productivity by the mutants obtained by the mutagenesis. The most potent lysine excretor, SR-V-1263 mutant, which produced approximately 2.5 mg/ml of lysine in the screening medium B, was obtained. Lysine formed by the mutant was identified by automatic amino acid analyzer: its retention time was essentially the same as that of the authentic lysine under the

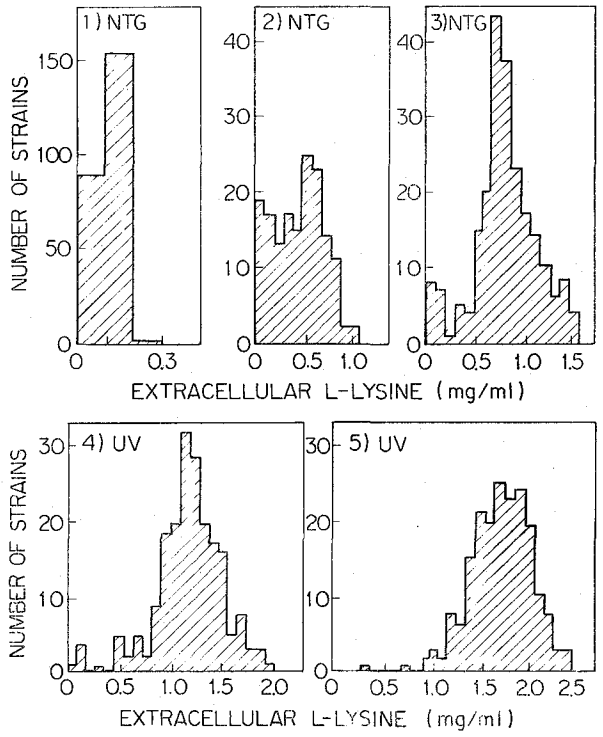

Fig. 1. Lysine production by SAEC-resistant mutants of *Candida pelliculosa*. The resistant mutants induced by the first mutation were grown in the screening medium A,[16] and the mutants obtained by the subsequent mutations were cultivated in the medium B.[16] The cultures were carried out in a test tube at 28°C for 96 hr with reciprocal shaking. Lysine was determined by the acid ninhydrin method.[19]

conditions used (35.3 min). The identity was confirmed also by paper chromatography with the following solvent systems: phenol-ethanol-water-ammonia (150:40:10:1, v/v/v/v) (Rf 0.57) and phenol solvent (Rf 0.35). Lysine excreted was quantitatively decarboxylated with L-lysine decarboxylase from *B. cadaveris*, indicating that the L-enantiomer is exclusively produced.

SR-V-1263 mutant could grow well in the presence of a high concentration of SAEC (0.5 % or 25 mM) as shown in Fig. 2, though the wild-type strain could not grow until at least 72 hr after inoculation.

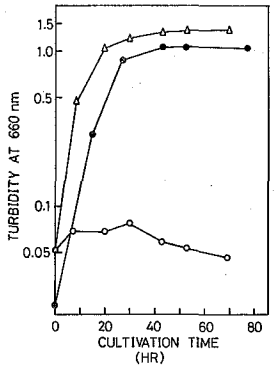

Fig. 2. Effect of SAEC on growth of strain SR-V-1263 and the wild-type strain of *Candida pelliculosa*. A 0.1-ml aliquot of the seed suspension was inoculated into the Lingens-Oltmanns' minimum medium[20] supplemented with SAEC. (△), Wild-type strain (none); (○), wild-type strain (0.5 % SAEC); (●), strain SR-V-1263 (0.5 % SAEC).

Effect of α-Aminoadipate on Lysine Production

Effect of α-aminoadipate, a precursor of lysine, on lysine accumulation by SR-V-1263 mutant and by the wild-type strain was investigated. The addition of 0.3 % DL-α-aminoadipate stimulated about 60 % the accumulation of extracellular lysine by the mutant strain (Table I). Fig. 3 shows the effect of 0.3 % DL-α-aminoadipate on the extracellular and intracellular lysine level during cultivation of the mutant or the wild-type strain. The addition of α-aminoadipate stimulated the extracellular lysine accumulation by the mutant, but only slightly affected the in-

Table I. Effect of DL-α-Aminoadipate on Accumulation of Extracellular Lysine by Strain SR-V-1263

Fermentations were carried out 28°C for 96 hr in test tubes with rotary shaking. Extracellular lysine was determined manometrically with L-lysine decarboxylase.

	Conc. (%)	Cell Growth (Turbidity at 660 nm)	Extracellular lysine (mg/ml)
DL-α-Aminoadipate	0.1	7.27	2.64
	0.2	8.32	2.92
	0.3	8.10	3.18
	0.4	8.62	3.06
None	—	—	2.00

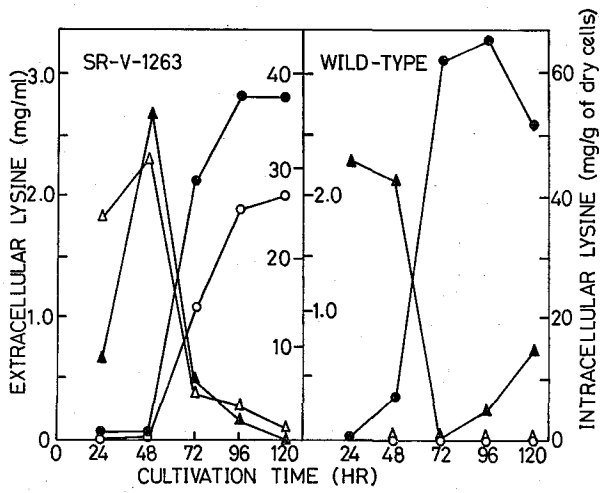

Fig. 3. Effect of DL-α-aminoadipate on accumulation of extracellular and intracellular lysine by strain SR-V-1263 and wild-type strain of *Candida pelliculosa*. The cells were cultured in the basal fermentation medium[16] in test tubes with or without 0.3% DL-α-aminoadipate under reciprocal shaking. Lysine was determined manometrically. (○), Extracellular lysine (none); (●), extracellular lysine (α-aminoadipate); (△), intracellular lysine (none); (▲), intracellular lysine (α-aminoadipate).

tracellular lysine level. The wild-type strain produced no appreciable amount of lysine extracellularly and intracellularly in the absence of α-aminoadipate, but the addition of the amino acid caused remarkable accumulation of extracellular and intracellular lysine: approximately 3 mg/ml of lysine was accumulated in the medium by the addition of α-aminoadipate 72–96 hr after inoculation.

Regulation of Homocitrate Synthase

Strain SR-V-1263 accumulated a high concentration of lysine in the medium and could grow well on the SAEC-medium (Fig. 2), suggesting that a regulatory enzyme in lysine biosynthesis are modified by the mutation to lose sensitivity to SAEC. We also found that incorporation of [1-^{14}C]-acetate into lysine in the wild-type strain was effectively inhibited by L-lysine and L-SAEC, but not in SR-V-1263 cells (E. Takenouchi, H. Tanaka and K. Soda, unpublished data). Thus, overproduction of lysine by the mutant strain may be attributed to release of a regulation of lysine biosynthesis. To confirm this, we examined the effect of L-lysine and L-SAEC on homocitrate synthase in cell-free extracts of the wild-type and the mutant strain.

L-Lysine and L-SAEC significantly inhibited homocitrate synthase activity of the wild-type strain (Table II). Both amino acids, however, showed only little effect on the enzyme of the mutant: the enzyme was inhibited by 16 % even in the presence of 1 mM L-lysine (Table II). The results indicate that the mutation site of SR-V-1263 mutant is homocitrate synthase, though the enzyme of the wild-type strain is controlled by both effectors. Therefore, overproduction of lysine

Table II. Effect of L-Lysine and L-SAEC on Homocitrate Synthase Activity in Cell-free Extracts of The Wild-type Strain and SR-V-1263 Mutant
The cell-free extracts were prepared as described under "EXPERIMENTAL PROCEDURES". The enzyme activity was assayed by the DTNB-method.

	Inhibition of homocitrate synthase (%)		
	Conc. (mM)	Wild-type strain	SR-V-1263 mutant
L-Lysine	0.1	24	10
	0.5	37	12
	1.0	57	16
L-SAEC	0.1	22	0
	0.5	24	0
	1.0	40	2

by the mutant strain is attributed to release of a feedback inhibition to homocitrate synthase by L-lysine.

Dissimilation of Lysine

To examine the lysine-dissimilating ability of the mutant and the wild-type strains, they were grown in the medium supplemented with L-lysine as a sole nitrogen source, and their growth were compared. Both strains could grow well on the lysine-medium and showed the similar growth on the minimum medium containing 0.1 % ammonium sulfate or 0.1 % L-lysine as a sole nitrogen source, respectively, though could not utilize lysine as a sole carbon source (Fig. 4). The mutant strain also showed almost similar growth to that of the wild-type strain on the lysine-medium, although the mutant grew a little slowly. We observed that [^{14}C]-L-lysine was transported into the mutant cells less rapidly than into the wild-type cells. This phenomenon may be attributable to a high level of intracellular lysine

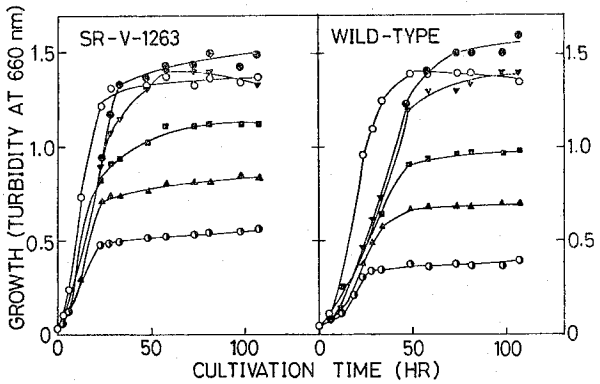

Fig. 4. Dissimilation of lysine by strain SR-V-1263 and wild-type strain of *Candida pelliculosa*. A 0.1-ml aliquot of the seed suspension was inoculated into the Lingens-Oltmanns' minimum medium[20] supplemented with L-lysine as a sole nitrogen source. (○), 0.1 % $(NH_4)_2SO_4$; (◑), 0.01% L-lysine; (▲), 0.025 % L-lysine; (■), 0.05 % L-lysine; (▼), 0.1 % L-lysine; (●), 0.2 % L-lysine.

in the mutant strain. It is likely that SR-V-1263 mutant can dissimilate lysine as well as the wild-type strain. Thus, overproduction of lysine by the resistant mutant is not ascribed to its inability of degrading intracellular lysine.

DISCUSSION

It is in general more difficult to mutate yeasts than bacteria because the vegetative cells of their most species are in a diploid form. The cells of some strains of the genus *Candida, e.g., C. pelliculosa* and *C. guilliermondii* var. *membranaefaciens* have been reported to be in a haploid form.[22,23] Tsukada and Sugimori[24] have induced auxotrophic mutants from *C. pelliculosa* by ultraviolet irradiation with a high mutation rate. In the present study SAEC-resistant mutants were efficiently induced by the mutagenesis.

Almost all the resistant mutants obtained excreted lysine in the medium. The most potent producer, strain SR-V-1263, accumulated 2.2-2.5 mg/ml of lysine in the screening medium (Fig. 1). DL-α-Aminoadipate, a precursor of lysine, stimulated extracellular accumulation of lysine by the mutant strain, though did not affect the intracellular lysine level. In the wild-type strain the precursor caused marked extracellular accumulation of lysine. It was, however, reported that *Saccharomyces cerevisiae* and *Torulopsis utilis* grown in the presence of α-aminoadipate or α-ketoadipate intracellularly accumulated as much as 16–20 % of their dry weight as lysine, but lysine was not excreted into medium.[25-27] Haidaris and Bhattacharjee[28,29] have reported that SAEC-resistant mutants of *S. cerevisiae* accumulate lysine extracellularly but the lysine concentration is very low. Our results are not consistent with their observations. Probably these two strains are different considerably in permeability of lysine: in *C. pelliculosa* lysine may penetrate through the cell membrane more rapidly. Tsukada and Sugimori[30] also described that *S. cerevisiae* is less capable of excreting metabolites into medium. Therefore, it is likely that *C. pelliculosa* is preferable to *S. cerevisiae* for potential lysine production.

The wild-type strain markedly excreted lysine into the medium when grown in the α-aminoadipate-medium, also suggesting that the mutant and the wild-type strain are not different in permeability of lysine.

To make clear the mechanism of overproduction of lysine by SR-V-1263 we investigated the effect of L-lysine and L-SAEC on homocitrate synthase of the mutant and the wild-type strain. The enzyme catalyzes the first step of lysine biosynthesis in yeasts and molds: condensation of α-ketoglutarate with acetyl-CoA to yield homocitrate. The enzyme has been reported to be feedback-controlled by lysine in *S. cerevisiae*,[31-35] *Neurospora crassa*[36] and *Saccharomycopsis lipolytica*.[37] In the present study we found that the enzyme activity in the cell-free extract of SR-V-1263 mutant was less sensitive than the wild-type enzyme to the inhibition by L-lysine and L-SAEC. This indicates that the mutation site of the mutant strain is homocitrate synthase, and that L-SAEC acts as a false feedback inhibitor of the enzyme. This analog also inhibits aspartokinase of *Escherichia coli*[38] and homocitrate synthase of *S. cerevisiae*.[35]

To study an alternative mechanism for overproduction of lysine by the mutant, the lysine dissimilation by the mutant cells was examined. The cells could utilize lysine effectively as a sole nitrogen source as well as the wild-type cells. This shows that lysine overproduction by the mutant is not ascribable to its inability of degrading intracellular lysine. Gaillardin and Heslot,[39] however, reported that certain mutants of *Saccharomycopsis lipolytica* defective in their lysine-dissimilating systems accumulated some lysine intracellularly.

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